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Epidermal growth factor (EGF) receptor is expressed in many normal tissues, including mammary epithelium. Overexpression or mutation of the EGF receptor causes neoplastic transformation in many cell types. Several studies have implicated the EGF receptor as a good prognostic marker in breast cancer, but its role in the etiology and progression of this malignancy is still under dispute. We propose to utilize a genetic approach to investigate this issue, by developing transgenic mice in which the EGF receptor gene in the mammary gland will be inactivated at the onset of first lactation. The construct for gene targeting was made. After introducing the construct in ES cells by electroporation and G418 selection, we have several positive clones for ES cell injection and chimeric mice from some of the positive clones. We hope to generate the transgenic mouse model next year so that we will be able to study the question whether the EGF receptor plays a functional role in the etiology and progression of mammary carcinoma. The answer to this question would indicate the feasibility of using the EGF receptor as a target in breast cancer therapy.

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FOREWORD

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Introduction

Breast cancer has been the leading cause of death among non-smoking women and thus has been the focus of intensive research. For the last decade or so, many researchers have concentrated on understanding the molecular basis of breast cancer. Since the epithelial cells of the breast are regulated by a variety of hormones and growth factors, it appears that abnormal hormonal milieu might be one of the critical factors in the development of breast cancer. EGF (epidermal growth factor) is a growth-stimulating factors and act as an autocrine and paracrine growth factor. The expression of both EGF and is positively regulated by estrogen and progesterone receptors [1].

EGF binds to EGF receptor and both epithelial and stromal cells of breast carcinoma express EGF receptor. The EGF receptor is a 170 kDa transmembrane glycoprotein, which belong to the tyrosine kinase receptor family. The role of the EGF receptor in breast cancer has been studied in great detail over the last decade, but it still remains under debate [2]. More than 10,000 breast cancer patients have been assayed for EGF receptor [2]. The expression of EGF receptors on normal breast epithelial cells is low and elevated expression of EGF receptors occurs in about 40% of primary breast cancers. There is a clear reverse relationship between EGF receptor expression and estrogen receptor expression, as well as between the expression of EGF receptor and progesterone receptor [3]. High levels of EGF receptor are associated with poor tumor differentiation, high tumor grade, aneuploidy and high rate of cell proliferation [4]. There is still no consensus, however, on the significance of EGF receptor in breast cancer prognosis or its correlation with the relapse-free survival and overall survival [5,6]. In node-positive patients, the EGF receptor appears to be a good prognostic marker, but in the node-negative patients, the EGF receptor does not appear to have a prognostic value [7,8].

EGF receptor, is detected in a large variety of tissues with the exception of hemopoietic cells. Overexpression of either the ligand of the EGF receptor or the receptor leads to the transformed phenotype in many different cell types [9-15].

The development of the transgenic mouse and gene knockout techniques provides an exceptional opportunity to elucidate the role of the EGF receptor signal pathway in mammary gland cancer [16]. Mouse models have already proven to be useful in mammary gland cancer studies. Thus, overexpression of the ligand of EGF receptor in the mammary gland of transgenic mice under the control of a strong MMTV (mouse mammary tumor virus) promoter or a matollothionein promoter results in a much higher rate of mammary neoplasia [17-19]. Neoplastic transformation of mammary glands in transgenic mice was also observed when c-myc was overexpressed from the MMTV or WAP (whey acidic protein) promoter [20,21].

The role of the EGF receptor in mammary physiology has been indicated by the analysis of a mutant mouse strain, waved-2 [22]. This strain contains a single nucleotide alteration of the EGF receptor gene at position 743, resulting in a valine to glycine substitution in a highly conserved region of the EGF receptor tyrosine kinase domain. The mutant EGF receptor lacks the high

affinity ligand binding and the rate of ligand-dependent internalization is slower than with the wild-type EGF receptor. The phenotype of the waved-2 mouse includes a smaller size of the mammary gland with a reduction of milk in the duct and a less pronounced secretary vacuolation within lobules, as compared to the wild-type mouse. However, it is still unclear whether the abnormal mammary gland of waved-2 mice is due to a developmental problem or to the impaired function of the EGF receptor.

Three groups have generated null mutations of the EGF receptor in mouse by homologous recombination in embryonic stem (ES) cells [23-25]. The phenotype of the null mutation in mouse turned out to be different in different genetic backgrounds. However, no female mice live long enough to develop mature mammary gland. Thus, the null mutation of the EGF receptor in mouse does not provide us with a model to study the role of the EGF receptor in mammary cancer. Therefore, we need to generate a mutant mouse lacking EGF receptor specifically in the mammary gland. Recent success of cre-loxP mediated gene knockout technique [26], which allows one to change the gene of interest in a tissue-specific manner, provides us with the tool to generate mice lacking EGF receptor only in the mammary gland. This mutant mouse will be our model to study the role of the EGF receptor in mammary cancer. We will evaluate the effect of the EGF receptor on the incidence of mammary tumors induced by chemical carcinogenesis. Analysis of this mutant mouse should reveal the role of the EGF receptor in the etiology and progression of breast cancer and will indicate the significance of the EGF receptor as a therapeutic target.

Body

1) Construction of the targeting vector for EGF receptor gene. (Task 1 in statement of work)

The targeting vector was finished last year as show in figure 1.

2) Cloning of the promoter region of WAP (white acid protein) gene and construction of WAP-*cre*. (Task 2 in statement of work)

We have cloned the promoter region of WAP last year and made the construct containing 3.5 kb WAP promoter and *cre* gene. After we made the construct, Jackson Lab has obtained the license from DuPont to sale transgenic mouse containing *cre* gene, including WAP-*cre* mouse, which is the same one that we plan to generate. Therefore, in stead of generating our own strain of mouse, we will purchase WAP-*cre* mouse from Jackson Lab when we need it.

3) Electroporation of ES cells with EGF receptor construct. (Task 3 in statement of work)

We have introduced the EGF receptor targeting vector we constructed into ES cells by electroporation. After ten days of G418 selection, we have isolated about 500 colonies. Each individual clone was expanded and the DNA from each clone is isolated for Southern blot analysis. The probe we used for Southern blot analysis is a ~500 bp fragment 5'-end outside targeting construct. After analyzing more than 400 individual clones, we identified 11 positive ones. We expanded the ES cells from those 11 positive clones for more DNA to confirm those ES cells had gone through the correct DNA recombination. We also use the *neo* gene as well as a 3'-end fragment as probes to determine the correct recombination. Southern bolt analysis reveals several positive ones.

4) Electroporation of ES cells with the *cre* gene to remove the *neo* gene cassette. (Task 3 in statement of work)

One of positive clones was selected to remove the *neo* gene cassette. The ES cells was electroporated with a plasmid containing *cre* gene under the control of CMV promoter. About 20 G418 negative clones were picked up. However, Southern blot analysis revealed that all of them were type II deletion (figure 2), and we expected to get 50% type I deletion and 50% type II deletion. We further tried several times with the same result. After attending the "Mouse Molecular Genetics" meeting, we found out some other labs have the same problem as we have. In order to solve the problem, we took several approaches. We used a different promoter, such as PGK, to express *cre* gene in ES cells and lowered the amount of the *cre* gene construct for eletroporation. After several trials, we were able to resolve the problem. Now we have both type I and type II deletion of ES cells. From the meeting, we have also learned that we do not have to generate both type I and type II deletion for our study

to understand the role of EGF receptor in mammary gland tumor. Recently, many labs have shown transgenic mice containing cre recombinase is sufficient to delete the exon of gene of interest to more than 80%. Apparently, it becomes not necessary for us to generate type II deletion mice for our studies and we will only need to generate type I homozygous mice that carrying two copies of loxP sites flanking exon 1 of EGF receptor. Therefore, it will save a lot of time to generate mice in our studies.

5) Generating mouse containing loxP sites flanking exon 1 of EGF receptor gene. (Task 5 in statement of work)

To generate mouse containing loxP sites flanking exon 1 of EGF receptor, we injected ES cells with type I deletion to generate chimeric mice. Recent studies have shown that transgenic mice carrying a *cre* gene under the control of EIIa promoter are able to delete sequence at the zygote stage [27]. Several other labs have used this strain of mice to delete the *neo* gene cassette successfully. Therefore, we are also going to try it. In other words, we will generate mice with our ES containing the *neo* gene cassette, and then will cross with EIIa-*cre* mouse, which is also available at Jackson Lab, to remove the *neo* gene cassette. We have generated some chimeric mice and are waiting for germ-line transmission.

Conclusions:

This year, we have run into some problems but we have resolved it. At the same time we have found ways to save time for our project from studies of other labs. It appears that we are a little behind our project according to our proposal; however, we will be able to catch up next year since we will save time in generating mice that we are going to use in our studies. We do not have any data related to breast cancer now, but we are moving along with our project. Next year we hope we will have the transgenic mouse to start our studies related to breast cancer.

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Appendices

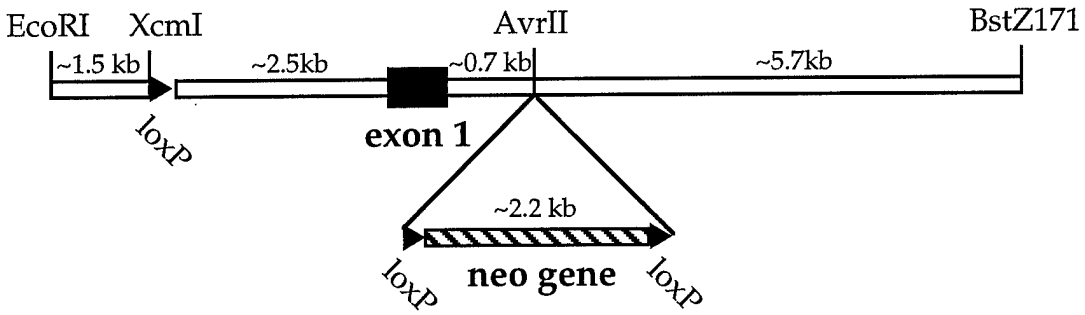

Figure 1. EGF receptor gene targeting construct

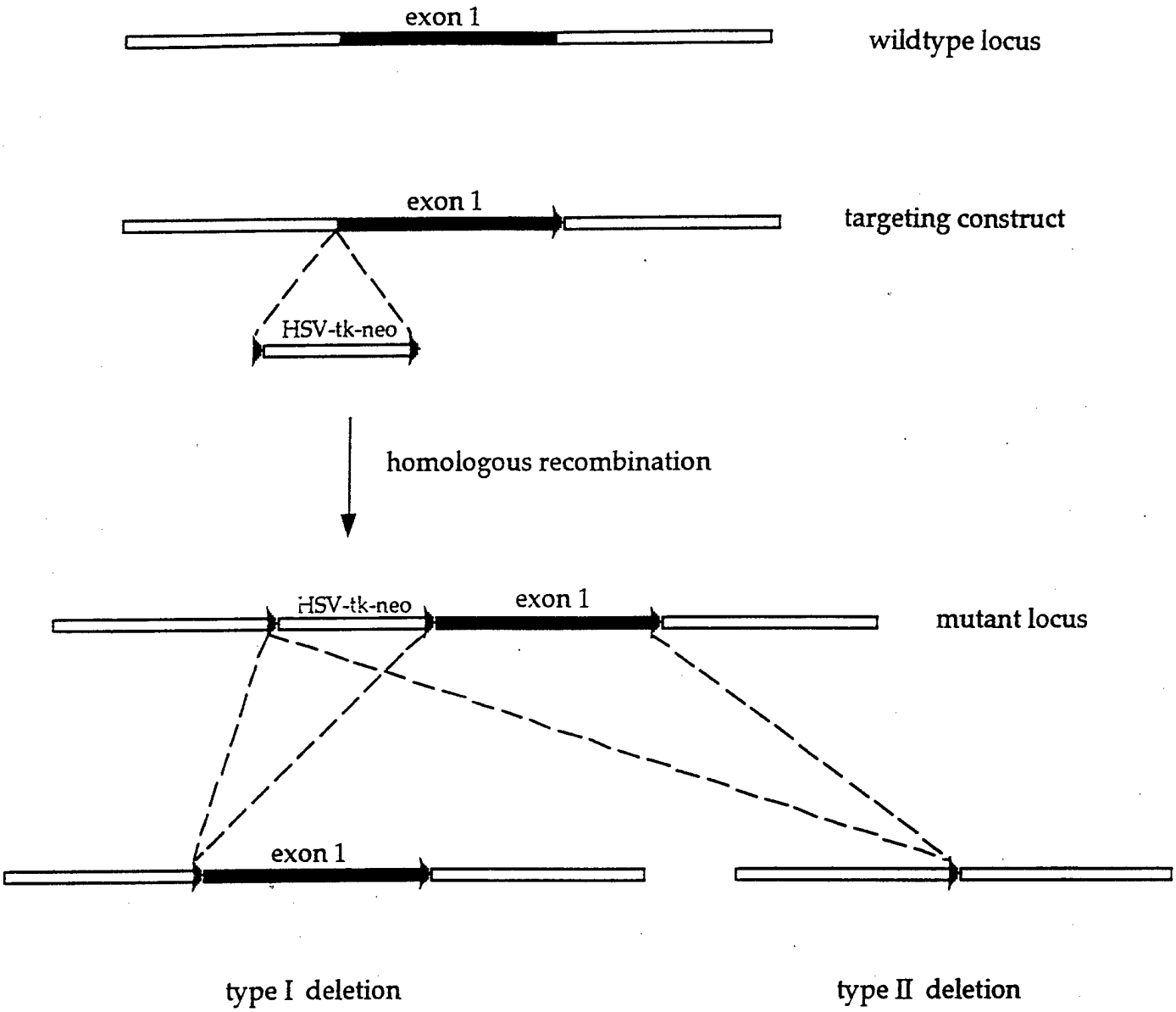

Figure 2. Strategy to generate genes flanking by loxP sites in ES cells.
Genetic locus, targeting construct and mutant locus as well as two types of cre-mediated deletions are shown.
The loxP site: ▶ .